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Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells

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Embryonic stem (ES) cells are clonal cell lines derived from the inner cell mass of the developing blastocyst that can proliferate extensively in vitro and are capable of adopting all the cell fates in a developing embryo. Clinical interest in the use of ES cells has been stimulated by studies showing that isolated human cells with ES properties from the inner cell mass[1,2] or developing germ cells[3] can provide a source of somatic precursors. Previous studies have defined in vitro conditions for promoting the development of specific somatic fates, specifically, hematopoietic, mesodermal, and neurectodermal[4–7]. In this study, we present a method for obtaining dopaminergic (DA) and serotonergic neurons in high yield from mouse ES cells in vitro. Furthermore, we demonstrate that the ES cells can be obtained in unlimited numbers and that these neuron types are generated efficiently. We generated CNS progenitor populations from ES cells, expanded these cells and promoted their differentation into dopaminergic and serotonergic neurons in the presence of mitogen and specific signaling molecules. The differentation and maturation of neuronal cells was completed after mitogen withdrawal from the growth medium. This experimental system provides a powerful tool for analyzing the molecular mechanisms controlling the functions of these neurons in vitro and in vivo, and potentially for understanding and treating neurodegenerative and psychiatric diseases.

To understand the developmental transitions of stem cells and fulfill their clinical promise, it must be possible to control the differentiation of ES cells into specific cell fates. Neurons, astrocytes, and oligodendrocytes have been derived from ES cells[7–10]. The neurotransmitters, dopamine and serotonin, have important functions in the etiology and treatment of neurodegenerative[11] and psychiatric diseases[12–15]. The neurons that synthesize these transmitters are generated by similar developmental signals around the boundary between the midbrain and the hindbrain.

To assess the developmental progression of ES cells in vitro, we examined the appearance of CNS- and midbrain-specific gene products in a modification of our previous work where CNS stem cells were derived from ES cells[10]. The differentiation involves several steps: the generation of embryoid bodies (EBs; stage 2), the use of a defined medium to select for CNS stem cells (stage 3), the proliferation of CNS stem cells in the presence of mitogen, basic fibroblast growth factor (bFGF; stage 4), and the differentiation of the stem cells by removal of the mitogen (stage 5; Fig. 1A).

The successful conversion of ES cells into midbrain dopaminergic (DA) neurons in vitro should be dependent on the induction of the same genes that are expressed in CNS stem cells and neurons in vivo. The *OTX* homeobox genes (*OTX1* and *OTX2*) are widely expressed at early stages of neurectoderm differentiation[16]. *OTX2* is expressed throughout the epiblast and subsequently restricted to anterior neurectoderm, where it is required for development of the forebrain and midbrain. The homolog *OTX1* is first expressed in the neurectoderm in the dorsal telencephalon, and interactions between these two *OTX* genes are thought to specify the development of the midbrain and hindbrain[17]. *OTX2* is expressed in the undifferentiated (stage 1) ES cells and present at lower levels at stage 2 and 3 of differentiation. *OTX1* is not expressed at high levels until stage 3 (Fig. 1B).

Several genes (*Pax2*, *Pax 5*, *Wnt1*, *En1*, *Nurr1*) have been identified that control differentiation of dopaminergic and serotonergic neurons in the midbrain and hindbrain[18,19]. PCR analysis detected expression of these genes at stages 3 and 4 of ES cell differentiation. After stage 4, the bFGF-expanded cells are morphologically uniform and express nestin, an intermediate filament protein characteristic of CNS stem cells (Fig. 2A)[20,21]. These results suggest that ES cells show a progressive restriction to mesencephalic and metencephalic CNS stem cells.

Identification of neurons synthesizing neurotransmitters expected for midbrain and hindbrain fates would lend support to this conclusion. Tyrosine hydroxylase (TH) is the rate-limiting enzyme for the biosynthesis of dopamine and a marker of ventral midbrain neurons. No TH expression was detected at stages 1–4 of ES cell culture (data not shown). However, the stage 4 cells may be primed for induction of TH, but still require time and appropriate conditions to express differentiated neuronal features. Differentiation (stage 5) was promoted by using conditions known to promote neuronal differentiation from the proliferating precursor state[22–24]. TuJ1, an antibody directed against the neuron-specific β-III tubulin bound many cells with a clear neuronal morphology (Fig 2B). Of the total cell population, 71.9 ± 6.9% were TUJ1$^+$, and 6.9% ± 1.5% of the TUJ1$^+$ cells were also TH$^+$ (these numbers are the average ± s.e.m. of three independent experiments, n > 40 microscopic fields, for a total containing 15.7×10^3 neurons). The

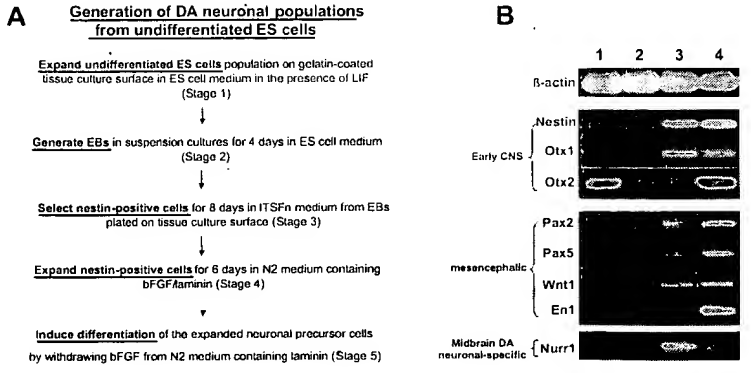


Figure 1. ES cells progressively differentiate into mesencephalic stem cells. (A) General scheme of ES cell culture. (B) Differentiated ES cells express genes characteristic of midbrain fates. Semi-quantitative RT-PCR analysis of the early CNS, mesencephalic, and midbrain-specific regulatory genes at different stages of ES cell culture. Numbers at the top of the panel designate stages of culture defined in (A).

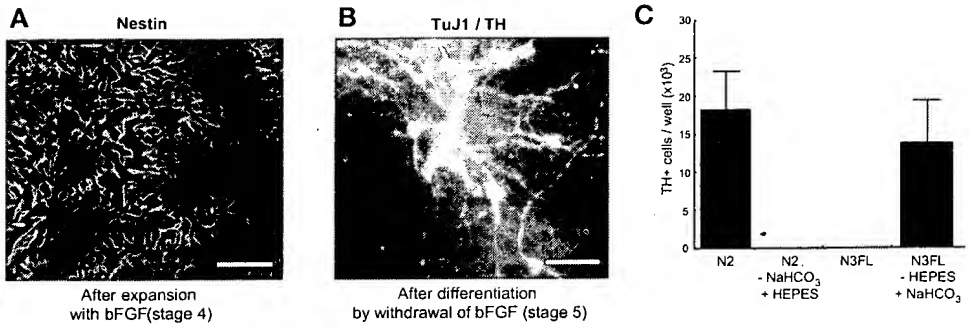


Figure 2. Mesencephalic precursors differentiate into TH⁺ neurons. (A) ES cell progeny at stage 4 express nestin, an intermediate filament protein expressed in CNS stem cells but not in neurons and glia (scale bar = 20 μm). (B) ES cell progeny at stage 5 differentiate into TH⁺ neurons. Removal of the mitogen initiates differentiation of the nestin-positive cells into neurons recognized by the antibody TuJ1 that binds to a neuron-specific tubulin. A subset of the neurons expresses the enzyme tyrosine hydroxylase (TH), which is required for the production of catecholamine neurotransmitters (scale bar = 20 μm). (C) The effect of HEPES buffer on differentiation of TH⁺ cells. N3FL is a medium used for ES culture[10].

neuronal differentiation is as efficient as has been previously achieved from primary CNS stem cells[22,25]. This result suggests that the ES-derived nestin-positive population is dominated by CNS stem cells and differentiates into midbrain neurons.

We found that the method of EB formation and the composition of growth medium exert a clear effect on the yield of TH⁺ neurons. The significant percentage of TH⁺ neurons among the differentiated ES cell progeny contrasts with previous studies in which no appreciable numbers of TH⁺ cells were observed[10]. The current procedure was achieved by two modifications that optimized neuronal differentiation and TH expression. In this work ES cells were first dissociated into single cells to obtain a more uniform procedure for EB formation. As a result of this modification the yield of nestin-positive cells, as determined after stage 3, was increased by 3.5-fold. The second modification was to use medium without HEPES during stages 4 and 5 of culture. HEPES inhibits differentiation of TH⁺ neurons (Fig. 2C). This modification of the culture conditions leads to the efficient production of TH⁺ neurons.

Sonic hedgehog (SHH), FGF8, and ascorbic acid (AA) increased the yield of ES-derived TH⁺ neurons. FGF8 and SHH have been previously shown to promote ventral midbrain fates in neural plate explant[26], therefore we hypothesized that the addition of these factors at specific stages during an in vitro ES cell differentation might also increase the proportion of DA and serotonergic neurons in our culture system. As shown in Figure 3A, the SHH receptors (smoothened, Smo, and patched, Ptc), a downstream transcription factor (Gli1), the FGF8 receptor (FGFR3), and the ligands (SHH and FGF8) are expressed during ES cell differentiation. Combined treatment with SHH/FGF8 during stage 4 leads to a greater than twofold increase in the number of TH⁺ cells (Fig. 3B; 15.4 ± 2.4% of the TuJ1⁺ neurons; value of three independent experiments, n > 40 fields containing 15.7×10^3 neurons, $P < 0.01$). Application of SHH/FGF8 at earlier (stage 2 or 3) or later (stage 5) stages had no effect on the number of TH⁺ neurons (data not shown). When applied as single factors at stage 4, SHH and FGF8 were both significantly less effective than when added in combination.

The cAMP analog, dibutyryl cAMP, and AA have been previously implicated in promoting DA neuron yield from primary CNS cultures[27,28]. Whereas dibutyryl cAMP was ineffective alone or in combination with SHH and FGF8, treatment with AA during stage 5 led to a significant increase in TH⁺ cell yield (Fig. 3B). The percentage of ES-derived TH⁺ neurons after treatment with SHH/FGF8 during stage 4 and AA during stage 5 reached 33.9 ± 5.5% of the neurons (n > 40 fields containing 4.2×10^3 neurons) which corresponds to more than 20% of the total cell population. Importantly, we found that the population of TH⁺ cells was maintained when stage 4 cells treated with SHH and FGF8 were passaged before differentiation.

To further characterize the ES-derived TH⁺-cell population we performed double immunohistochemistry for TH and (1) dopamine-β–hydroxylase (DBH), a marker of noradrenergic neurons (2) γ-aminobutyric acid (GABA), a marker for inhibitory neurons, and (3) serotonin, a transmitter found in many ventral hindbrain neurons. No coexpression of TH with any of these markers was observed, suggesting that the TH⁺ cells are DA neurons (data not shown).

The production of dopamine is a definitive measure of the identity of a dopaminergic neuron. Reverse-phase high-performance liquid chromatography (RP-HPLC) was used to measure dopamine release directly (Fig. 3C). The dopamine peak can be readily separated and the sensitivity of the procedure repeatedly confirmed with control samples. Neurons were differentiated in stage 5 for six days, and medium conditioned for 48 h was tested. Consistent with the increase in the number of TH⁺ neurons, the dopamine level was increased more than twofold in cultures treated with SHH/FGF8/AA (n = 3, $P < 0.05$). Elevated potassium was used to determine if dopamine could be released by depolarization. When Hank's balanced salt solution (HBSS) was applied to the cells for 15 min, 165.7 ± 23.4 pg ml^{-1} dopamine was detected. SHH/FGF8/AA did not change dopamine levels. However, dopamine levels were elevated when the neurons were depolarized with elevated potassium (56 mM), and the medium collected after 15 min (416.6 ± 7.2 pg ml^{-1} in the controls and 918.4 ± 123.2 pg ml^{-1} of dopamine in the SHH/FGF8/AA-treated cultures). These data demonstrate that ES-derived TH⁺ cells secrete significant levels of dopamine when depolarized.

Electrophysiology was used to determine if ES-derived neurons were functionally active. When current was injected into individual patch-clamped neurons, sustained trains of action potentials, characteristic of mature neurons, were routinely observed (n = 54; Fig. 4A). The cells also responded to direct application of the neurotransmitters GABA and glutamate (Fig. 4B, C). After 13 days of differentiation at stage 5, the neurons showed spontaneous activity that was almost completely blocked by tetrodotoxin (TTX), an inhibitor of action potentials (Fig. 4D, E). Biocytin labeling demonstrated that neurons exhibiting TTX-sensitive spontaneous activity were TH⁺ (Fig. 4F, G). This demonstrates that TH⁺ cells receive synaptic input and suggests that this system can be used to define synaptic control of dopamine release.

In addition to specification of midbrain DA precursors, SHH is also important for the generation of hindbrain serotonergic neurons[26]. As shown in Figure 5, in untreated cultures 0.8 ± 0,1% (n = 56, fields containing 6.2×10^3 neurons) of all TuJ1⁺ neurons were serotonin-positive. However, addition of SHH/FGF8 during stage 4 increased the serotonergic population by 14-fold (11.0 ± 0.5%, n = 56 fields containing 6.2×10^3 neurons, $P < 0.01$ of all TuJ1⁺ cells). Serotonin and TH were not coexpressed. Interestingly, application of SHH alone promoted serotonergic fates to an extent similar to the combined treatment (Fig. 5B). These results are in agreement with

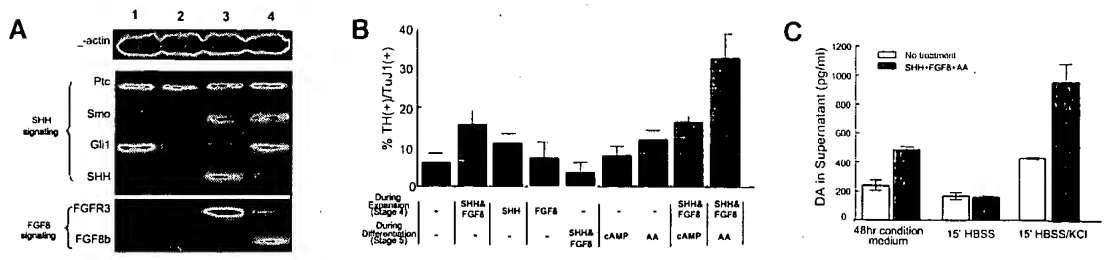
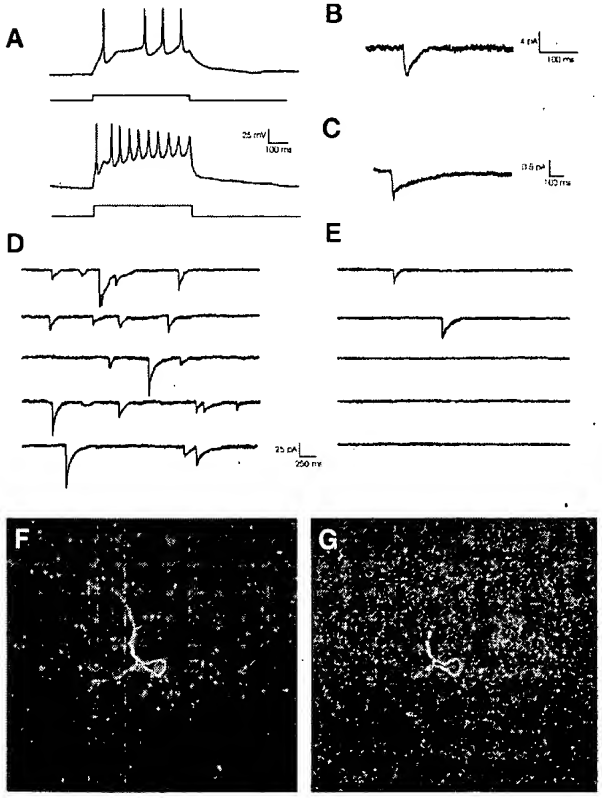


Figure 3. The effect of external signals on the yield of neurons that are TH⁺ and secrete dopamine. (A) Expression of genes in the SHH and FGF8 signal pathways. Semiquantitative RT-PCR analysis at different stages of cell culture. Numbers at top of the panel designate stages of the culture; see Figure 1A. (B) The yield of TH⁺ neurons is expressed as a percentage of TuJ1⁺ neurons. SHH (500 ng ml⁻¹), FGF8 (100 ng ml⁻¹), cAMP (1 mM), and AA (200 μM) were added at different stages of ES cell development, as shown. (C) The effect of treatment with SHH, FGF8, and AA on maturation of DA neurons as measured by dopamine release. The RP-HPLC determination of dopamine concentration is shown in medium (N2) conditioned for 48 h (left), in HBSS conditioned for 15 min (center), and in HBSS + 56 mM KCl conditioned for 15 min (right).

findings in neural plate explant cultures suggesting that SHH can act alone to specify the precursors for serotonergic neurons [26]. The efficient induction of serotonergic neurons by SHH suggests that these differentiation conditions support hindbrain serotonergic fates.

It has been shown that ES cells can differentiate into neurons and glia in vitro[7,10] and in vivo[8,9,29]. However, no systematic derivation of midbrain and hindbrain neurons from ES cells has been reported. Here we found that catecholaminergic and serotonergic neurons can be efficiently derived from ES cells. HEPES buffer addition during stage 4 and stage 5 was incompatible with DA differentiation of ES cells. At present the mechanism of this inhibitory effect is not known. SHH, FGF8, and AA also enhance the differentiation to dopaminergic and serotonergic fates. Less than 5% of the neurons express TH when mesencephalic cells are directly differentiated without expansion in the precursor state[30]. When E12 rat mesencephalic precursors were first expanded in bFGF, 25% of the neurons were TH⁺ (ref. 24). This ES cell system gives access to different stages of development leading to >30% of TH⁺ neurons, the highest yield ever obtained either in vitro or in vivo.



The development of cell therapy is a potential advantage of the directed differentiation of ES cells into DA neurons. Although later precursors such as CNS stem cells can also be expanded and differentiated into DA neurons, these have only a limited ability to expand in vitro and to adopt a dopaminergic fate; moreover, these cells do not produce more DA neurons in response to SHH and FGF8 treatment (our unpublished observation). The ES cells, on the other hand, proliferate without limit, respond to signaling molecules, and are easily accessible to genetic manipulation. We anticipate that the efficiency of the system described here can be further improved by generating ES cell lines expressing regulatory genes such as *Nurr1* known to control commitment to DA fate in vivo[31]. We also expect that the purification of a homogeneous midbrain population can be achieved by genetic methods. Although studies in Parkinsonian rodents are needed to further assess the function and safety of ES cell-derived DA neurons in vivo, our data demonstrate that the neurons derived from ES cells produce dopamine, respond to neurotransmitters, and exhibit spontaneous synaptic activity.

The central finding of this study is that midbrain and hindbrain neurons can be generated in unlimited numbers from ES cells. For every 3×10^6 of ES cells, we obtain 21×10^6 neurons and 7×10^6 DA neurons. Numerically, two to three TH⁺ cells are harvested at stage 5 for every undifferentiated ES cell plated at stage 1. This represents the sum total of all parameters of proliferation, cell death, and selective differentiation throughout the five culture stages. There are 3×10^4 DA neurons in the rat substantia nigra, and 10^3 grafted DA cells lead to behavioral recovery in a rat model of Parkinson's disease[25]. Genetic manipulation may now be used to obtain pure populations of the DA neuron suitable for grafting and other experiments relevant to the etiology and therapy of Parkinson's disease.

The dopamine- and serotonin-synthesizing neurons are two ventral fates generated in vivo, anterior and posterior of the isthmic organizer, a source of signaling molecules that control the differentiation

Figure 4. Synaptic properties of ES-derived TH⁺ neurons. (A) Action potential spiking behavior. Following 13 days of differentiation (stage 5), depolarization causes the cell to fire an initial action potential followed by a few others at low frequency. With increasing amounts of depolarization the neuron will fire a train of action potentials at a higher frequency. This behavior was common in the cultures and is characteristic of mature neurons. (B) GABA application to the dendrites of a neuron leads to an inward current. (C) Glutamate application to the dendrites of a neuron leads to an inward current. (D) Spontaneous activity in TH⁺ neurons recorded in voltage clamp mode. (E) Tetrodotoxin (TTX) blocks spontaneous activity in TH⁺ neurons. The activity in the same cell as shown in (D) was diminished when action potentials were blocked with 1 μM TTX. Note that the TTX blocks almost all spontaneous activity, indicating that most of the activity is synaptic release of transmitter evoked by spontaneous action potential firing in presynaptic neurons. (F, G) The cell recorded in (D) and (E) is TH⁺. The cell tracer biocytin was introduced through the recording electrode, and confocal images are presented showing the colocalization of TH (F) and biocytin (G) (Scale bars = 20 μm).

when more than two groups were involved. If data were not normally distributed, a nonparametric test (Mann–Whitney U-test) was used for the comparisons of results. Data were expressed as mean ± s.e.m.

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Genome-directed primers for selective labeling of bacterial transcripts for DNA microarray analysis

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DNA microarrays have the ability to analyze the expression of thousands of the same set of genes under at least two different experimental conditions[1]. However, DNA microarrays require substantial amounts of RNA to generate the probes, especially when bacterial RNA is used for hybridization (50 µg of bacterial total RNA contains approximately 2 µg of mRNA)[2]. We have developed a computer-based algorithm for prediction of the minimal number of primers to specifically anneal to all genes in a given genome. The algorithm predicts, for example, that 37 oligonucleotides should prime all genes in the *Mycobacterium tuberculosis* genome. We tested the usefulness of the genome-directed primers (GDPs) in comparison to random primers for gene expression profiling using DNA microarrays. Both types of primers were used to generate fluorescent-labeled probes and to hybridize to an array of 960 mycobacterial genes. Compared to random-primer probes, the GDP probes were more sensitive and more specific, especially when mammalian RNA samples were spiked with mycobacterial RNA. The GDPs were used for gene expression profiling of mycobacterial cultures grown to early log or stationary growth phases. This approach could be useful for accurate genome-wide expression analysis, especially for in vivo gene expression profiling, as well as directed amplification of sequenced genomes.

We developed a computer algorithm to define the minimal number of oligonucleotides of a given length capable of priming all genes within any genome. Using the genome sequence of *Mycobacterium tuberculosis*, we applied the algorithm, setting the oligonucleotides length at eight or seven bases, and requiring 100% coverage of the 3,924 open reading frames (ORF) in the genome[3] (Fig. 1A). The search was limited to the first 500 bp of each complementary sequence of each ORF to generate long probes for efficient hybridization. The priming efficiency of the mycobacterial genome-directed primers (mtGDPs; 37 primers) was compared to the priming efficiency of seven- or six-nucleotide random primers in a standard reverse transcription reaction. Probes generated from the same mycobacterial RNA (log phase cultures) using mtGDPs or random primers were hybridized simultaneously to the same slides, and the signal intensities of the 960 arrayed genes were calculated and compared[4,5]. There was a high correlation level between the signals of both probes for the whole array of genes ($r = 0.97$). Additionally, signals generated by mtGDPs were significantly higher than random primer-generated signals ($P < 0.05$) (Fig. 1B, C). Signal intensities

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